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(54) Title: TYROSINE PHOSPHATASE IA-2, GAD AND ROTAVIRUS VP7 IMMUNITY

(57) Abstract

The present invention provides T cell epitopes from IA–2. The present invention also relates to the use of these epitopes in diagnosis and therapy. The present invention further provides rotavirus vaccines.

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Tyrosine IA-2, GAD and rotavirus VP7 phosphatase immunity

FIELD OF THE INVENTION

The present invention relates to T-cell epitopes of tyrosine phosphatase IA-2 and to methods involving the use of these epitopes.

BACKGROUND OF THE INVENTION

Activation of T-cells requires recognition by T-cell receptors of specific, antigenic peptides complexed to major histocompatibility complex (MHC) molecules on the surface of target or antigen-presenting cells (1). Such T-cell epitopes, "restricted" by the MHC molecule, are potential tools for the diagnosis, monitoring and therapy of infectious, autoimmune and neoplastic disorders.

The recently-identified pancreatic islet autoantigen in type 1 diabetes, IA-2, is a 106 kD member of the protein tyrosine phosphatase family (2,3) and an integral membrane protein of neuroendocrine secretory granules (4). Circulating autoantibodies that recognise predominantly the cytoplasmic domain of IA-2 can be detected in up to 88% of people with recently-diagnosed type 1 diabetes and in about half of islet-cell antibody (ICA)-positive, first-degree type 1 diabetes relatives in whom they indicate high risk for clinical disease (5, 6). The cytoplasmic domain of IA-2 has 80% sequence identity with another tyrosine phosphatase, IAR (7), also known as IA-2 β (8) or phogrin (9), which also reacts with antibodies in type 1 diabetes (10). T-cell proliferative responses to IA-2 were reported to be increased in at-risk relatives and in people with recently-diagnosed type 1 diabetes (11). T-cell epitope peptides in autoantigens have potential diagnostic and therapeutic applications and may hold clues to environmental agents that could trigger or exacerbate autoimmune disease. The present inventors have identified T-cell epitope peptides within the intracytoplasmic domain of IA-2 and examined them for sequence similarities with microorganisms and dietary proteins as a basis for molecular mimicry.

SUMMARY OF THE INVENTION

Accordingly in a first aspect the present invention consists in a tyrosine phosphatase IA-2 T-cell epitope, the T-cell epitope having a sequence included within or consisting of a sequence selected from the

group consisting of IA-2 aa685-700 ANMDISTGHMILAYME, IA-2 aa713-728 WQALCAYQAEPNTCAT, IA-2 aa745-760 PYDHARIKLKVESSPS, IA-2 aa787-802 LSHTIADFWQMVWESG, IA-2 aa793-808 DFWQMVWESGCTVIVM, IA-2 aa799-814 WESGCTVIVMLTPLVE, IA-2 aa805-820 VIVMLTPLVEDGVKQC, IA-2 aa841-856 SEHIWCEDFLVRSFYL, IA-2 aa845-860 WCEDFLVRSFYLKNVQ, IA-2 aa847-862 EDFLVRSFYLKNVQTQ, IA-2 aa889-904 DFRRKVNKCYRGRSCP, IA-2 aa919-934 YILIDMVLNRMAKGVK, IA-2 aa959-974 FEFALTAVAEEVNAIL and conservative substitutions therein.

Sequences from the related tyrosine phosphatase IAR with high % identity and homology to the IA-2 T-cell epitopes, which are themselves potential T-cell epitopes, are also included within the present invention. Accordingly in a second aspect the present invention consists in a T-cell epitope, the T-cell epitope having a sequence included within or consisting of a sequence selected from the group consisting of IAR aa721-736 SNMDISTGHMILSYME (88% identity, 88% homology), IAR aa749-764 WEALCAYQAEPNSSFV (69%, 75%), IAR aa781-796 TYDHSRVLLKAENSHS (56%, 69%), IAR aa823-838 LPATVADFWQMVWESG (81%, 88%), IAR aa829-844 DFWQMVWESGCVVIVM (94%, 94%), IAR aa835-850 WESGCVVIVMLTPLAE (88%, 94%), IAR aa841-856 VIVMLTPLAENGVRQC (81%, 94%), IAR aa877-892 SEHIWCEDFLVRSFYL (100%, 100%), IAR aa881-896 WCEDFLVRSFYLKNLQ (94%, 100%), IAR aa883-898 EDFLVRSFYLKNLQTN (88%, 100%), IAR aa925-940 DFRRKVNKCYRGRSCP (100%, 100%), IAR aa955-970 YVLIDMVLNKMAKGAK (81%, 100%), IAR aa995-1010 FEFALTAVAEEVNAIL (100%, 100%) and conservative substitutions therein.

In a preferred embodiment of the present invention the T-cell epitope has a sequence included within or consisting of the sequence VIVMLTPLVEDGVKQC.

In a further preferred embodiment the T-cell epitope has the sequence VIVMLTPLVED.

As will be recognised by those skilled in the art modifications may be made to the peptides of the present invention while still retaining function. Such modifications include having amino acid substitutions compared to the native IA-2 or IAR sequence but which retain certain structural and functional characteristics. These modifications include

additions, deletions and substitutions, in particular conservative substitutions. It is intended that peptides including such modifications are within the scope of the present invention.

Whilst the concept of conservative substitution is well known in the field for the sake of clarity the types of substitutions envisaged are set out below.

Original Residue	Exemplary Substitutions	Preferred Substitutions
Ala (A)	val; leu; ile	val
Arg (R)	lys; gln; asn	lys
Asn (N)	gln; his; lys; arg	gln
Asp (D)	glu	glu
Cys (C)	ser	ser
Gln (Q)	asn	asn
Glu (E)	asp	asp
Gly (G)	pro	pro
His (H)	asn; gln; lys; arg	arg
Ile (I)	leu; val; met; ala; phe; norleucine	leu
Leu (L)	norleucine, ile; val; met; ala; phe	ile
Lys (K)	arg; gln; asn	arg
Met (M)	leu; phe; ile;	leu
Phe (F)	leu; val; ile; ala	leu
Pro (P)	gly	gly
Ser (S)	thr	thr
Thr (T	ser	ser
Trp (W)	tyr	tyr
Tyr (Y)	trp; phe; thr; ser	phe
Val (V)	ile; leu; met; phe ala; norleucine	leu

Another type of modifications of the peptides envisaged include, but are not limited to, modifications to side chains, incorporation of

unnatural amino acids and/or their derivatives during peptide synthesis and the use of crosslinkers and other methods which impose conformational constraints on the peptides.

Examples of side chain modifications contemplated by the present invention include, but are not limited to, modifications of amino groups such as by reductive alkylation by reaction with an aldehyde followed by reduction with $NaBH_4$; amidation with methylacetimidate; acylation with acetic anhydride; carbamoylation of amino groups with cyanate; trinitrobenzylation of amino groups with 2,4,6-trinitrobenzene sulphonic acid (TNBS); acylation of amino groups with succinic anhydride and tetrahydrophthalic anhydride; and pyridoxylation of lysine with pyridoxal-5'-phosphate followed by reduction with $NaBH_4$.

The guanidine group of arginine residues may be modified by the formation of heterocyclic condensation products with reagents such as 2,3-butanedione, phenylglyoxal and glyoxal.

The carboxyl group may be modified by carbodiimide activation *via* O-acylisourea formation followed by subsequent derivitisation, for example, to a corresponding amide.

Tryptophan residues may be modified by, for example, oxidation with N-bromosuccinimide or alkylation of the indole ring with 2-hydroxy-5-bitrobenzyl bromide or sulphenyl halides. Tyrosine residues on the other hand, may be altered by nitration with tetranitromethane to form 3-nitrotyrosine derivative.

Modification of the imidazole ring of a histidine residue may be accomplished by alkylation with iodoacetic acid derivatives or N-carbethoxylation with diethylpyrocarbonate.

Examples of incorporating unnatural amino acids and derivatives during peptide synthesis include, but are not limited to, use of norleucine, 4-amino butyric acid, 4-amino-3-hydroxy-5-phenylpentanoic acid, 6-aminohexanoic acid, t-butylglycine, norvaline, phenylglycine, ornithine, sarcosine, 4-amino-3-hydroxy-6-methylheptanoic acid; 2-thienyl alanine and/or D-isomers of amino acids.

The peptides of the present invention may be derived from IA-2 and IAR. It is, however, preferred that the peptides are produced synthetically using methods well known in the field. For example, the peptides may be synthesised using solution synthesis or solid phase synthesis as described,

for example, in Chapter 9 entitled "Peptide Synthesis" by Atherton and Sheppard which is included in a publication entitled "Synthetic Vaccines" edited by Nicholson and published by Blackwell Scientific Publications. Preferably a solid phase support is utilised which may be polystyrene gel beads wherein the polystyrene may be cross-linked with a small proportion of divinylbenzene (e.g. 1%) which is further swollen by lipophilic solvents such as dichloromethane or more polar solvents such as dimethylformamide (DMF). The polystyrene may be functionalised with chloromethyl or anionomethyl groups. Alternatively, cross-linked and functionalised polydimethyl-acrylamide gel is used which may be highly solvated and swollen by DMF and other dipolar aprolic solvents. Other supports can be utilised based on polyethylene glycol which is usually grafted or otherwise attached to the surface of inert polystyrene beads. In a preferred form, use may be made of commercial solid supports or resins which are selected from PAL-PEG, PAK-PEG, KA, KR or TGR.

In solid state synthesis, use is made of reversible blocking groups which have the dual function of masking unwanted reactivity in the α-amino, carboxy or side chain functional groups and of destroying the dipolar character of amino acids and peptides which render them inactive. Such functional groups can be selected from t-butyl esters of the structure $RCO\text{-}OCMe_3\text{-}CO\text{-}NHR$ which are known as t-butoxy carboxyl or ROC derivatives. Use may also be made of the corresponding benzyl esters having the structure $RCO\text{-}OCH_2\text{-}C_6H_5$ and urethanes having the structure $C_6H_5CH_2OCO\text{-}NHR$ which are known as the benzyloxycarbonyl or Z-derivatives. Use may also be made of derivatives of fluorenyl methanol and especially the fluorenyl-methoxy carbonyl or Fmoc group. Each of these types of protecting group is capable of independent cleavage in the presence of one other so that frequent use is made, for example, of BOC-benzyl and Fmoc-tertiary butyl protection strategies.

Reference also should be made to a condensing agent to link the amino and carboxy groups of protected amino acids or peptides. This may be done by activating the carboxy group so that it reacts spontaneously with a free primary or secondary amine. Activated esters such as those derived from p-nitrophenol and pentafluorophenyl may be used for this purpose. Their reactivity may be increased by addition of catalysts such as 1-hydroxybenzotriazole. Esters of triazine DHBT (as discussed on page

215-216 of the abovementioned Nicholson reference) also may be used. Other acylating species are formed in situ by treatment of the carboxylic acid (i.e. the Na-protected amino acid or peptide) with a condensing reagent and are reacted immediately with the amino component (the carboxy or C-protected amino acid or peptide). Dicyclohexylcarbodiimide, the BOP reagent (referred to on page 216 of the Nicholson reference), O'Benzotriazole-N, N, N'N'-tetra methyl-uronium hexaflurophosphate (HBTU) and its analogous tetrafluroborate are frequently used condensing agents.

The attachment of the first amino acid to the solid phase support may be carried out using BOC-amino acids in any suitable manner. In one method BOC amino acids are attached to chloromethyl resin by warming the triethyl ammonium salts with the resin. Fmoc-amino acids may be coupled to the p-alkoxybenzyl alcohol resin in similar manner. Alternatively, use may be made of various linkage agents or "handles" to join the first amino acid to the resin. In this regard, p-hydroxymethyl phenylactic acid linked to aminomethyl polystyrene may be used for this purpose.

In a third aspect the present invention consists in a method of assessing the risk of an individual developing type 1 diabetes. The method comprises measuring responsiveness of T cells of individuals at-risk, either by being a relative of an individual with type 1 diabetes, or with other immune markers of sub-clinical disease eg circulating autoantibodies to islet antigens, or by being exposed to a potential environmental trigger factor (eg non-exclusively virus, dietary agent, toxin). T-cell responses to the peptides are measured non-exclusively by the number of cells with activation markers or with specific levels of activation markers (eg CD69, CD44, CD25), numbers of cells with specific cytokines (eg interferon-γ, IL-4, IL-2, IL-10, TNF-α and others) or the levels of cytokines produced in or secreted by the cells, or by proliferation of T cells. All measurements are in comparison to T-cell responses from the same individual without the peptides, and in comparison to responses from healthy controls. Elevated (or changing) responses on any of these, or other appropriate, measures of T-cell responses to the peptides on one (or multiple) occasion/s may indicate the level of risk of type 1 diabetes.

In a fourth aspect the present invention consists in a method of therapy for the prevention of the onset of type 1 diabetes in those assessed to

be at-risk. The assessment of risk may be determined either by the method of the present invention, or by T-cell responses to whole molecules or peptides from other type 1 diabetes-associated autoantigens (eg insulin, proinsulin, glutamic acid decarboxylase), and/or by the presence of antibodies to islet autoantigens.

The therapeutic use of the peptides is to induce tolerance to protect against or ameliorate the symptoms associated with type 1 diabetes, by the oral, aerosol, intranasal or other mode of administration of the peptides to mucosal surfaces, or by other appropriate methods of administration eg non-exclusively transdermally, subcutaneous or intravenous. The presentation of soluble protein antigen to mucosal surfaces, classically via the oral route, results in selective suppression of antigen-specific T-cell responses, and has been associated with the deviation of immunity away from pro-inflammatory Th1 T-cell responses to antibody (Th2) responses. Regulatory cells, and, at higher antigen doses, T-cell anergy and T-cell deletion, have been shown to be induced. Aerosol inhalation of an autoantigen (insulin)in an animal model for type 1 diabetes was effective in reducing islet cell pathology and incidence of diabetes(12).

In a fifth aspect the present invention consists in a method of therapy for the prevention of the re-establishment of type 1 diabetes in those who have received a pancreatic or islet-cell transplant to alleviate their pre-existing type 1 diabetes. The therapy is intended to prevent, reduce or otherwise ameliorate the development of T-cell responses to the autoantigens in the graft, which could lead to its destruction. The method of administration is as in the above paragraph.

The present inventors have identified T-cell epitope peptides in the intracytoplasmic domain of the type 1 diabetes autoantigen, tyrosine phosphatase IA-2, whose sequence analysis suggests that immunity to rotavirus (whose VP7 sequence mimics epitopes in both IA-2 and GAD) could predispose to type 1 diabetes by activating crossreactive T cells.

The findings set out herein suggest that RV infection may trigger or exacerbate islet autoimmunity, on the HLA-DR4 background. As rotavirus vaccines currently undergoing trials are live viruses containing the VP7 leader sequence, these findings have implications for development of safe rotavirus vaccines to protect against islet autoimmunity and prevent the development of type 1 diabetes.

Accordingly in a sixth aspect the present invention consists in a vaccine composition for use in raising an immune response in a subject directed against rotavirus, the composition comprising a plurality of antigens including at least one rotavirus VP7 antigen wherein the sequence of the rotavirus VP7 antigen is modified such that at least one of the sequences which mimic one or more of the epitopes selected from the group consisting of ILLQYVVKSF, ILLNYVRKTF and VIVMLTPLVED are deleted or modified such as to remove or ameliorate mimicry.

In a preferred embodiment of this aspect of the present invention the composition includes at least one attenuated strain of rotavirus, the rotavirus nucleic acid encoding VP7 being modified such that the expressed VP7 antigen is modified such that at least one of the sequences which mimic one or more of the epitopes selected from the group consisting of ILLQYVVKSF, ILLNYVRKTF and VIVMLTPLVED are deleted or modified such as to remove or ameliorate mimicry.

In a further preferred embodiment the sequence which mimics ILLQYVVKSF or ILLNYVRKTF is deleted.

The following is merely one example of the modifications which may be made to the sequences to remove or ameliorate mimicry are as follows:-

VP7aa	17	18	21	24	40	44	45
(RV G3, strain P)	I	L	Y	K	I	L	S
Change to	A	A	A	H	A	A	F

aa 17: A, a small hydrophobic residue, replaces the P1 anchor for binding to DR4, DQ8

aa 18: A, a small hydrophobic residue replaces a T-cell receptor contact residue (TCR-CR)

aa 21: A, a small hydrophobic residue replaces a TCR-CR for both DR4, DQ8

aa 24: H, a weaker basic residue, replaces K which is conserved in all rotavirus strains and in both GAD65 and GAD67 epitopes

aa 40: A, a small hydrophobic residue, replaces P1 anchor for binding to DR4, DQ8

aa 44: A, a small hydrophobic residue replaces a TCR-CR for DR4, and the P4 anchor for binding DQ8

aa 45: F, a bulky hydrophobic residue, present in other G1 VP7 sequences in this position, replaces the P6 anchor for binding to DR4, and a TCR-CR for DQ8.

The sequences rotavirus VP7 from various strains are known. These sequences are provided in Reddy et al. Nucleic Acids Res. 17, 449 (1989), Green et al. Virology 161, 153-159 (1987), and Richardson et al. J. Virol. 51, 860-862 (1984). the disclosure of these references is incorporated herein by reference.

In a seventh aspect the present invention consists in a ligand for antigen-specific T lymphocytes, the ligand comprising a multimeric peptide-MHC complex in which the peptide is selected from the group consisting of ANMDISTGHMILAYME, WQALCAYQAEPNTCAT, PYDHARIKLKVESSPS, LSHTIADFWQMVWESG, DFWQMVWESGCTVIVM, WESGCTVIVMLTPLVE, VIVMLTPLVEDGVKQC, SEHIWCEDFLVRSFYL, WCEDFLVRSFYLKNVQ, EDFLVRSFYLKNVQTQ, DFRRKVNKCYRGRSCP, YILIDMVLNRMAKGVK, FEFALTAVAEEVNAIL, SNMDISTGHMILSYME, WEALCAYQAEPNSSFV, TYDHSRVLLKAENSHS, LPATVADFWQMVWESG, DFWQMVWESGCVVIVM, WESGCVVIVMLTPLAE, VIVMLTPLAENGVRQC, SEHIWCEDFLVRSFYL, WCEDFLVRSFYLKNLQ, EDFLVRSFYLKNLQTN, DFRRKVNKCYRGRSCP, YVLIDMVLNKMAKGAK, FEFALTAVAEEVNAIL and conservative substitutions therein.

In a preferred embodiment the peptide is VIVMLTPLVEDGVKQC or VIVMLTPLVED.

In a preferred embodiment of this aspect of the invention the multimer is a dimer or tetramer.

In a further preferred embodiment the ligand is provided with a detectable label.

The detectable label may be any one of a number of any such labels well known in the art such as biotin, a fluorophor, or radioisotope.

Further information regarding such ligand can be found in Altman *et al.*, Science 274; 94-96, 1996 and Dunbar *et al.*, Curr. Biol. 8 413-416, 1998. The disclosure of these references is incorporated herein by reference.

The ligand of this aspect of the present invention can be used in assessing the risk of an individual of developing type 1 diabetes. The

method would involve measuring the numbers of antigen-specific T lymphocytes in a sample from the individual. Where the ligand is provided with a label this could be done by flow cytometry. Further, as would be readily appreciated the ligand of the present invention could be used *in vivo* for imaging.

Throughout this specification the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated element, integer or step, or group of elements, integers or steps, but not the exclusion of any other element, integer or step, or group of elements, integers or steps.

The disclosure of all references referred to throughout this specification are incorporated herein by reference.

DETAILED DESCRIPTION

In order that the nature of the present invention may be more readily understood preferred forms thereof will now be described with reference to the following examples and Figures.

Figure legend

Fig 1: Summary of identified T-cell epitope peptides in tyrosine phosphatase IA-2. Bolded boxes contain sequences common to overlapping epitope peptides; unbolded boxes contain epitope peptides presented by both DR3-DQ2 and DR4-DQ8 haplotypes; stippled boxes contain epitope peptides presented only by the DR4-DQ8 haplotype.

Fig 2: Proliferative T cell response to IA-2 peptides in subjects at risk for type 1 diabetes; DR4DQ8 homozygous subjects (2a); DR3DQ2 homozygous subjects (2c); heterozygous subjects (2b).

Fig 3: T cell responses to peptides in tyrosine phosphatase IA-2 for each group of subjects.

Fig 4: RV Ab and islet Ab responses over 5 years in sibs at-risk for type 1 diabetes. A=sib 1, female, HLA-DR3,4; B=sib 2, male, HLA-DR1,4. Arrows indicate RV infections, dotted lines indicate antibody cut-offs.

RESEARCH DESIGN AND METHODS

Subjects for epitope study

Peripheral blood was obtained from six at-risk, islet cell antibody (ICA) positive first-degree relatives of people with type 1 diabetes (4 male, 2 female, mean age 28.5±15.0, range 10-50) and two healthy control subjects (2 males, ages 30, 48). Subjects were selected for type 1 diabetes-associated HLA haplotypes, ie DR4-DQ8 homozygous (two at-risk relatives, one control), DR3-DQ2 homozygous (two at-risk relatives, one control) and DR4-DQ8/DR3-DQ2 heterozygous (two at-risk relatives). All relatives had antibodies to IA-2. Within 14 months of the study, both DR4-DQ8 homozygous relatives developed clinical type 1 diabetes and the first-phase insulin release in response to intravenous glucose in both DR3-DQ2 homozygous relatives fell to below the first percentile, indicating imminent clinical disease. The study was approved by the Ethics Committee, and conducted with informed consent.

Subjects for epidemiologic study

360 infants in the Australian BabyDiab Study, with a parent or sibling with type 1 diabetes, had serum assayed every 6 months from birth for IAA, GADAb and IA-2Ab. Infants who developed diabetes (n=5, 2 male, at mean age 29.5±10.4 months) or had either >1 islet Ab or an Ab detected at >1 timepoint (n=19, 11 male) (group A) were studied for evidence of RV infections, together with 17 (9 male) unrelated age-, sex- and HLA class II-matched infants from the Study either without detectable islet Ab over the same period (n=10, 3 male) or with only one Ab transiently (n=7, 6 male) (group B). Thirteen siblings (8 male) of infants in group A were also studied to assess intra-familial transmission of RV. Acute- and convalescent-phase sera were collected from 10 children (5 male, mean age 11 months, range 6-38 months) hospitalised with serologically-proven RV gastroenteritis. These children had no first-degree relatives with type 1 diabetes. The studies were conducted with approval of human ethics committees.

Tissue typing

HLA alleles were typed by the standard microlymphocytotoxic method for all recognised HLA class I alleles. HLA-DR and DQ types were

determined by sequence-specific oligotyping, following the International Histocompatibility Workshop protocol.

Peptides

A set of 68 16-mer peptides was synthesised (Chiron Technologies, Melbourne, Australia). Sixty-two peptides overlapping by 10 aa spanned the cytoplasmic domain of human IA-2 (aa 601- 979). Six additional 16-mers (aa 713-728, 779-794, 795-810, 831-846, 845-860, 959-974) covered sequences predicted to bind to DR4(*0401) (13,14). Peptides were synthesised by Fmoc chemistry and solid phase synthesis, with free amino and free acid carboxy-termini, using base-labile or acid-labile resins as appropriate. Each peptide was dissolved in 100μl 40% acetonitrile in degassed phosphate buffered saline (PBS) and shaken at 4°C overnight, checked for solubility, sonicated in an immersion sonicator for up to 60 min at room temperature (RT) if necessary, then diluted to 1 mg/ml in PBS. Each peptide was dispensed into 12 wells of a sterile 96-well round-bottomed tissue culture tray (Linbro) and stored at -80°C.

HLA-DR4 binding

Peptide binding to purified DR4(*0401) was measured directly by a competition enzyme linked immunosorbant assay, as previously described (15,16).

T-cell proliferation assays

Peripheral blood mononuclear cells (PBMC) were separated from heparinised venous blood by Ficoll-Hypaque density centrifugation, washed twice in RPMI 1640 medium and diluted to 10^6 cells/ml in RPMI1640 medium containing 10% autologous serum, 20 mM Hepes and 10^{-5}M 2-mercaptoethanol (complete medium). Two x 10^5 cells were added in 200 µl of complete medium to each well of freshly-thawed, peptide-containing 96-well trays. Each peptide was tested at 10µg/ml in replicates of 12. The first row of each tray contained six wells without antigen (basal) and six wells with 1.8 Lyons flocculating units (Lfu)/ml of preservative-free tetanus toxoid (Commonwealth Serum Laboratories, Melbourne); the last row of each tray contained six wells with 0.18 Lfu of tetanus toxoid and six wells without antigen. After incubation for 6 days in 5% CO_2 at 37°C, 37 kBq

^{3}H-thymidine (ICN, 2.5 TBq/mmol) was added per well; the cells harvested semi-automatically seven hours later and ^{3}H-thymidine incorporation measured by liquid scintillation counting. As T-cell responses to peptides approximate a Poisson rather than a normal distribution, proliferation was expressed as the % positive of the 12 replicate wells. Positive wells were defined as having cpm >mean + 2 SD of the 12 basal wells for that plate. A T-cell response to a peptide was defined as positive wells $\geq$40%; this threshold was the mean + 2SD of the 136 responses of the controls to all peptides (mean 6%, SD 17%). T-cell epitopes were defined as being within peptides that elicited a response in two at-risk relatives with the same HLA haplotype, eg both DR4-DQ8 homozygotes, or one DR4-DQ8 homozygote and at least one DR3-DQ2/DR4-DQ8 heterozygote. The reproducibility of T-cell proliferation to tetanus (1.8 Lfu/ml) was tested by repeat assays weekly for four weeks in three subjects; intra-assay CVs ranged from 13.1 to 18.9 % and the inter-assay CV from 14.2 to 26.2%.

Antibody (Ab) assays

IAA

IAA were assayed by fluid-phase ^{125}I-insulin precipitation (17). Results were expressed as percent of total counts precipitated. The control range (mean + 2 SD) <5.5%, was derived from 190 healthy children (mean age 9.7; range 4.9-15.5 years). The inter-assay coefficient of variation (COV) is 16%.

GAD and IA-2Ab

GADAb and IA-2Ab were assayed by precipitation of 35S-methionine labelled recombinant human proteins and results were expressed as arbitrary units, described elsewhere (6). The control range for GADAb derived by receiver operator curve (ROC) analysis of 246 control subjects and 135 newly-diagnosed patients is <5U, with an inter-assay COV of 21%. In the international GADAb proficiency test #2, the assay scored 100% for sensitivity, specificity, validity and consistency. The control range for IA2Ab, derived by ROC analysis of 145 control subjects and 94 newly-diagnosed patients is <3 U, with an inter-assay COV of 34%.

Rotavirus IgA (RVA) and IgG (RVG) Ab

Levels of RVA and RVG were measured initially at a serum dilution of 1:100 by direct enzyme immunoassay (EIA), described previously for RVA in secretions (18,19). Sera were also tested at 1:500 as necessary. Optimal dilutions of reagents determined by chequerboard titration were dispensed in 100 ul aliquots. SA11 RV antigen and MA104 cell control antigen were prepared as previously (18). EIA antigens diluted in 0.06M sodium carbonate-bicarbonate buffer pH 9.6 were adsorbed to microtiter plate wells (NUNC Maxisorp) for 2 h at 37°C. After washing in phosphate-buffered saline containing 0.05% (v/v) Tween 20 (PBS-T20) and addition of sera diluted in PBS-T20 containing 0.5% (w/v) casein (PBS-T20-C), plates were held overnight at 4°C. Following washing, affinity-purified sheep anti-human IgA or IgG conjugated to horseradish peroxidase (Silenus, Australia) diluted in PBS-T20-C was added for 1.5 h at 37°C. Negative and positive controls, and colour development with TMB substrate, have been described (18). A standard human serum pool arbitrarily assigned to contain 20,000 units (U) RVG/ml and 30,000 U RVA/ml was titrated in doubling dilutions on each plate to construct a standard curve from which U/ml of RVG and RVA in test sera were determined. The inter-assay COV for both RVA and RVG was 20%. The positive/negative cut-off (241U) for RVA was determined from the mean+2SD in 25 cord sera. As all cord sera are RVG positive, the cut-off (550U) for RVG was the mean+2SD of the lower level in sera collected from 25 infants at 6 and 12 months of age, to allow for decay of transplacentally-acquired RVG. The specificity of these assays was shown previously (14,15).

CoxsackieB IgM Ab (CBVM)

CBVM were measured by EIA (16) in 82 sera, 47 with significant increases in RVA or RVG, as controls for specificity of association between RV and islet Ab. In young children the assay detects homotypic responses that become heterotypic (ie recognize multiple serotypes of CBV) with increasing age (20). Sera at 1:400 dilution were first screened against pooled antigens from CBV serotypes 4 and 5; positive sera were retested with individual antigens. The cut-off was the mean+3SD of 10 known negative serum samples/tray. The optimum serum dilution (1:400) was derived from previous titrations to 1:10,000, and specificity established by demonstrating

no crossreactivity with sera positive for antibodies to Epstein-Barr, measles, mumps and hepatitis A viruses, *Mycoplasma pneumoniae* and rheumatoid factor.

Thyroid peroxidase antibodies (TPOAb)

TPOAb were measured with the ELI test^{R} anti-TPO kit (Henning Berlin GMBH)

Anti-nuclear antibodies (ANA)

ANA were measured with the HEp 2000^{TM} fluorescent ANA-Ro test system (Immunoconcepts, Sacramento).

Database searches

Similarities to the sequences of epitope peptides or their common overlapping sequences were sought using FASTA 2 software. Databases searched were Genbank (GBTrans) (1997), Swissprot (1997), Protein Research Foundation of Japan (PRFJ) (1997) and Ooi Japan (OOIJ) (1983). No statistical significance was assigned to search results because the databases included many sequences homologous to IA-2, eg B220, CD45, IA-2 β, phogrin, IAR, and other tyrosine phosphatases. Infectious or dietary agents were selected on the basis of potential biological relevance, as in other studies (21), from the first 60 best matches in the PRFJ and OOIJ databases and from the first 100 in the larger GBTrans and Swissprot databases.

Statistical analysis

Any change ≥ 2 inter-assay COV between consecutive samples was considered to be significant, a standard practice for virological assays (18). Thus, a rise ≥ 32% for IAA, ≥ 42% for GADAb and ≥ 68% for IA-2Ab was considered significant; for RVA or RVG a rise of ≥ 40%, and for CBVM any increase above the cut-off, was considered indicative of infection during the preceding 6-month period. Either RVA or RVG was taken to indicate RV infection, as at-risk infants may have impaired IgA responses (22), and RVG does not rise immediately when pre-existing RVG is high (23,24).

A significant increase in any Ab was scored as 1, otherwise 0. Concordance (11 or 00) and discordance (10 or 01) for any islet Ab with any RV Ab was used for χ^2 analysis with Yates' correction to determine the

association over all samples in groups A and B. For greater statistical stringency, we employed a permutation analysis (25) in which the odds ratio (OR) was calculated as (11 x 00)/(10 x 01) for the association in each infant. The result was converted to $\log_{10}$, and the mean log OR of all 24 unrelated group A infants calculated. The distribution of the mean log OR expected if there was no association (null hypothesis) was derived by 1000 permutations of the positions of the scores of 1 and 0 for islet and RV Ab in each child, calculating the mean log OR at each permutation. The experimental mean log OR was then compared to the distribution of the mean log ORs from the permutations to determine the probability of association (25).

RESULTS

From 68 16-mer peptides encompassing cytoplasmic IA-2, 11 peptides (from aa 685, 713, 745, 787, 793, 805, 841, 845, 847, 919 and 959) elicited T-cell responses in relatives homozygous for DR4-DQ8 and two peptides (from aa 799 and 889) elicited responses in one DR4-DQ8 homozygous and one DR4-DQ8/DR3-DQ2 heterozygous relative (Figure 1, Figure 2). All these epitope peptides bound to HLA-DR4 (26) (Table 1). Five peptides (from aa 799, 805, 841, 847, 919) elicited responses in the DR3-DQ2 homozygous relatives, and the first four of these also in the matched control. Notably, peptide EDFLVRSFYLKNVQTQ (aa 847-862) elicited responses in both DR3-DQ2 and DR4-DQ8 homozygous controls, as well as in one DR3-DQ2 homozygous, one heterozygous and both DR4-DQ8 homozygous at-risk relatives. Peptide VIVMLTPLVEDGVKQC (aa 805-820) elicited a response in all at-risk relatives and in the DR3-DQ2 homozygous control; in each case it was the highest response (relatives, $86\pm20\%$ positive wells; control 100% positive wells). Alignment of the IA-2 epitope peptides with related sequences in human tyrosine kinase IAR (5) revealed identities of $86\pm12\%$ and similarities of $92\pm10\%$ (mean $\pm$ SD) (Table 2).

Epitope peptides of IA-2 shared identity/similarity with several environmental agents (Table 1). The dominant epitope peptide VIVMLTPLVEDGVKQC had sequence identities of 75-45% and similarities of 100-64% over 8-11 aa to sequences within the VP7 protein of rotavirus (serotype G3, strain P) and lesser identity to the G1 and G2 subtypes (Table 3). VIVMLTPLVEDGVKQC also has sequence identities with the capsid protein C of Dengue flavivirus, the major capsid protein of human

cytomegalovirus, the haemagglutinin proteins of canine distemper virus (known to infect humans) and the closely-related measles virus, and the E2 protein of hepatitis C virus. It also had 50% identity and 71% similarity over 14 aa with the HI 1338 protein of the bacterium Haemophilus influenzae. Most of the sequence similarities were in the region of overlap, VIVMLTPLVE (aa 805-814), with the preceding epitope peptide (aa 799-814). The rotavirus VP7 protein also had 75% identity and 92% similarity over 12 aa (aa 18-29) (or 75% and 100% over 9 aa) to GAD65 (aa 117-128), and GAD67 (aa 123-134) (Tables 4 & 5).

Peptide aa 685-700 had 56-71% identity and 78-86% similarity to the BTRF1 and BRRF2 proteins of Epstein-Barr virus, and 50% identity and 100% similarity over 10 aa to the genome polyprotein of rhinovirus 14, the common cold virus (Table 1).

Peptide aa 787-802 had 58% identity and 75% similarity over 12 aa to the M polyprotein precursor of hantavirus, and 71% identity and similarity over 7 aa to sequences within the genome polyprotein of other members of the flavivirus family, ie Japanese encephalitis, Kunjin, West Nile and Murray Valley encephalitis viruses. Most of the sequence similarities were in the region of overlap DFWQMVWESG (aa 793-802) with the succeeding epitope peptide (aa 793-808).

Peptide aa 841-856 had 64% identity and 82% similarity over 11 aa to NADH ubiqinone reductase proteins in wheat and broad beans, and epitope peptide aa 919-934 had 60% identity and 80% similarity over 10 aa to kappa casein in cow's milk. Most of the sequence similarities were in the region of overlap, EDFLVRSFYL (aa 847-856), with the two succeeding epitope peptides (aa 845-860, 847-856).

Peptide aa 919-934 had 63% identity and 88% similarity over 8 aa to the surface glycoprotein of Herpes simplex virus. Peptide aa 959-974 had 67% identity and 78% similarity over 9 aa to the major capsid protein of cytomegalovirus (HHV5) and Herpes saimiri virus (which can infect human lymphocytes), and 50% identity and 70% similarity over 10 aa to replication protein E1 of papilloma virus strains 28 and 18. It also had 45% similarity and 73% similarity over 11 aa to the E2L polyprotein of vaccinia and variola (HHV6) viruses. No sequence similarities were detected with the remaining three epitope peptides, aa 713-728, 745-760, 889-904.

In the epidemiologic study the first appearance or an increase in islet Ab (Table 6) was significantly associated by standard χ^2 test with an increase in RVA or RVG during the same 6-month period (χ^2_{YC} 13.2, $p<0.0003$ group A, χ^2_{YC} 7.7, $p<0.005$ group B). As χ^2 may be biased by high numbers of events the more stringent permutation analysis was then applied. The mean concordance of islet and RV Ab in serial samples from the 24 group A infants analysed by the log OR was 1.024. The mean log OR from 1000 permutations of 1 (significant increase) and 0 (no increase) scores per infant were normally distributed between –0.8 and 1.0 (Fig 3). The observed association was therefore confirmed as highly significant ($p<0.001$).

IAA, GADAb and IA-2Ab appeared at 10±21(mean+sd), 22±17 and 19±14 months respectively. Not all islet Ab appeared in all infants, but IAA first appeared with an increase in RV Ab in 13/21 (62%), GADAb in 10/20 (50%) and IA-2Ab 12/14 (86%) (eg Fig 4).

There was no association of CBVM with increases in islet Ab. Of 82 sera tested for both RV and CBVM, 35 had increases in islet Ab but only two of these, with GADAb, were also positive for CBVM (one of each serotype 4 and 5). In the 47 RV Ab positive sera, CBVM were detected to serotype 4 in four and to serotype 3 in one; in the RV negative sera, CBVM were detected to serotype 4 in one and serotype 5 in two.

As evidence for the specificity of the islet Ab response, TPO Ab measured in 27 concordant islet/RV Ab events were increased only once (from a raised level of 190 to 560 units), and were not increased in the non-concordant events; furthermore, ANA were not detected in concordant islet/RV Ab events.

There was no apparent cross-reactivity between islet antigens and RV at the Ab level. Thus, when four sera containing GADAb, IA-2Ab and RV Ab were adsorbed on high titre RV overnight and then retested, RVA and RVG decreased a mean of 57% and 40% respectively, whereas GADAb (7%) and IA-2Ab (0%) were unchanged.

In the group A infants, IAA, GADAb and IA-2Ab appeared or increased with repeated RV infections (Table 6). In the group B infants, transient Ab were IA-2Ab and IAA in 6/7 and 1/7 cases respectively. When RV Ab were compared in 52 concurrent samples from 13 paired at-risk siblings, infection occurred in both sibs 18 times, in neither 17 times, and in only one 17 times

(χ^2_{YC} 6.4, $p<0.02$) over the same 6 month period, ie 51% had concurrent infection.

The type 1 diabetes HLA-DR4 susceptibility haplotype was present in all infants (n=17) in whom any islet Ab increased above the cutoff only with RV infection and in all infants (n=10) in whom any islet Ab increased without RV infection. However, the HLA-DR3 susceptibility haplotype was present in only 4/17 (24%) of the former concordant compared to 7/10 (70%) of the latter discordant infants ($p<0.05$). HLA-DQ2-linked DR alleles (DR3 and DR7) were present in 6/17 (35%) concordant compared to 9/10 (90%) of discordant infants ($p<0.02$).

In the 10 unrelated children hospitalised with proven RV infection, GADAb were detected in one acute and another convalescent serum and IA2Ab in one acute and three convalescent sera (Table 7).

DISCUSSION

Thirteen peptides within the intracytoplasmic domain of IA-2, all of which could be presented by HLA-DR4 encoded by the DR4-DQ8 haplotype, elicited T-cell responses in at-risk relatives. The overlap of these peptides suggests nine epitopes. Five peptides, between aa 799-934, elicited responses in relatives bearing either the DR4-DQ8 or DR3-DQ2 susceptibility HLA haplotypes. The remaining peptides elicited responses only in relatives bearing the DR4-DQ8 haplotype. At least two sources of these epitopes are indicated by the very high degree of homology between the two tyrosine phosphatases, IA-2 and IAR.

Interestingly, four peptides elicited responses in the DR3-DQ2 homozygous control, and one of these four also in the DR4-DQ8 homozygous control. Other evidence demonstrates that T cells in normal individuals are capable of reacting to autoantigens (11,27-29). The important inference, however, is that these four epitope peptides (shared sequences VIVMLTPLVE, EDFLVRSFYL) should contain the strongest clues to crossreactive epitopes, eg in environmental agents that could trigger or exacerbate islet autoimmunity.

The contribution of environment to type 1 diabetes can be gauged from the lack of concordance for disease in the majority of identical twins (30). However, the environmental factors responsible remain enigmatic. Some viruses such as Coxsackievirus (31) and rubella (32), as well as the

rodenticide "Vacor" (33), directly damage pancreatic islet β-cells and are associated with β-cell autoimmunity, but such examples are rare, and evidence for persisting infection of β-cells is lacking (34). Infectious agents could also trigger β-cell autoimmunity indirectly (reviewed in 35), particularly by activating T cells crossreactive with islet proteins, a mechanism termed molecular mimicry.

Molecular mimicry has been proposed between the islet autoantigen glutamic acid decarboxylase 65 (GAD65) (amino acids, aa 257-273) and the P2C protein of Coxsackievirus B4, which share 59% identity and 76% similarity over 17 aa (36). This peptide from GAD65 elicits T-cell responses in humans with type 1 diabetes (29) and in the non-obese diabetic (NOD) mouse model (37). T-cell responses to Coxsackie virus B (strain unstated) have been reported in recently-diagnosed type 1 diabetes (21). However, evidence for mimicry is weak, as two overlapping GAD peptides that share the sequence don't elicit T-cell responses in the same individuals with diabetes (36), stronger responses occur in healthy controls (29), and other studies (38,39,40) have not found responses to the CBV-like sequence in GAD. On the other hand, increased IgM to CBV3 and 5 has been found in the sera of pregnant mothers whose infants subsequently developed diabetes (41,42), and CBV3, 4 and 5 infections have been associated with islet cell Ab in children and adolescents with first-degree relatives with diabetes (43).

Evidence for a role of viral infection close to diagnosis of type 1 diabetes is the finding that IgM responses to Coxsackievirus (44) and T-cell responses to both Coxsackievirus and adenovirus, but not to the Herpes viruses, or to mumps, polio, tick-borne encephalitis virus or rotavirus (21), were higher in people at diagnosis than in controls. The dominant IA-2 epitope peptide aa 805-820 has high identity and similarity over 8-11 aa to sequences within several viruses. The nonamer in this peptide predicted to bind to DR4 (14) is VIVMLTPLV. The most likely anchor residues for binding (Table 1; 10,11) are unbolded; the bolded residues are therefore most likely to be T-cell receptor contact residues (TCR-CR) potentially critical for molecular mimicry. The strongest similarity is with the VP7 protein of human rotavirus particularly (serotype 3, strain P, reovirus family, Table 2). VP7 contains the sequence IIVILSPLL (aa 41-49) with identical TCR-CR; although the anchor residues differ they are equally effective for DR4 binding (14). By using HLA-DQ8(*0302) binding peptides curated in the

MHCPEP Database (45) to derive a matrix for DQ8, two overlapping decamers in the same IA-2 region were also predicted to bind to DQ8 (Table 1), consistent with the high T-cell responses seen to this epitope. The first DQ8 frame, IIVILSPLLN, has 100% similarity to VP7 in its potential TCR-CR. VP7 is one of the two immunogenic proteins that confer serotype specificity and is currently being used by others to develop a rotavirus vaccine (46).

Jones and Crosby (21) noted a sequence similarity between GAD 65 (aa 108-137) and rotaviral VP7 protein, although they could not elicit increased T-cell responses to whole rotavirus (strain unstated) in people with recently-diagnosed type 1 diabetes. The cited GAD65 sequence contains a T-cell epitope peptide MNILLQYVVKSFDRST (aa 115-130, with 88% homology to GAD67, aa 121-136), in mice transgenic for human HLA-DR4 (47). We have identified this epitope (aa 115-129) in at-risk relatives and healthy controls homozygous for DR4-DQ8. The predicted DR4-binding nonamer within the GAD65 peptide is ILLQYVVKS, and for VP7 is ILLNYVLKS; in GAD 67 the equivalent region is ILLNYVRKT. GAD65 therefore has 100% similarity and identity with VP7 in the potential TCR-CR (Table 4). The region of VP7 containing both sequence similarities is immunologically interesting. It contains many hydrophobic potential anchor residues for HLA class II molecules, and an epitope for cytotoxic T cells in C57/Bl6 mice immunized with rotavirus (48,49), adjacent to the sequences with similarity to GAD65 and IA-2. The GAD and IA-2 similarities raise the interesting possibility that rotavirus infection could simultaneously activate T cells to two type 1 diabetes autoantigens (see also below).

Rotavirus is a major enteric pathogen of early childhood (50) that causes regular winter outbreaks of gastroenteritis in daycare centres. Children can have multiple infections by different serotypes. Early-age daycare was found to confer increased risk for type 1 diabetes (51), consistent with a link between rotavirus and type 1 diabetes. Furthermore, the most marked increase in type 1 diabetes incidence over the last decade (11%/yr) has occurred in the 0-4 year old age group(62).

VP7, the major outer capsid protein of RV, is an important determinant of virulence and induces virus-neutralizing antibodies (50). However, the elimination of RV following infection is predominantly due to T cells (52).

Proliferative CD4 T-cell responses have been detected in humans within 4-6 weeks following rotavirus re-infection (53). These CD4 T cells were of the CD45RA negative (memory), α4β7 integrin-high subset, indicating that gastrointestinal immune responses generate α4β7 positive T-cell memory. An interesting convergence is that GAD-responsive T cells from people with recently-diagnosed type 1 diabetes are α4β7 positive (84) and T cells in the early phase of insulitis in NOD mice are β7-integrin high (55). These data suggest that rotavirus-responsive CD4, α4 β7 positive T cells could migrate selectively to the islets. As the T-cell contact residues in the similar RV VP7 and IA-2 sequences appear to be identical (56), there is potential for molecular mimicry. All human RV serotypes in the Genbank database contain the GAD-related sequence (Table 4). These IA-2- and GAD-like sequences in VP7 span an epitope (aa 31-40) for cytotoxic T cells in C57/Bl6 mice immunized with RV (48,49), consistent with this region being strongly immunogenic. RV infection could therefore simultaneously activate T cells crossreactive to two islet autoantigens in genetically-susceptible infants.

In addition to molecular mimicry, RV may directly infect islets. This possibility is supported by reports of pancreatitis following rotavirus infection (57,58), and by the fact that rotavirus is a related to reovirus, which can infect human (59) and mouse (60) islets.

The similarities of the other viruses with peptide VIVMLTPLVEDGKQC and with the other IA-2 epitope peptides include anchor residues for DR4(*0401), but the potential TCR-CR are not quite as remarkable as for rotavirus.

IA-2 epitope peptide aa 919-934, as well as being similar to the surface glycoprotein of Herpes simplex virus, has 60% identity and 80% similarity over 10 aa, that include the predicted DR4 binding nonamer ILIDMVLNR, with bovine kappa casein YIPIQYVLSR (aa 26-35), although the similarity of the potential TCR-CR is only 40%. T-cell responses to whole casein have been reported in type 1 diabetes (61) but the role of bovine milk proteins as potential aetiologic agents in type 1 diabetes is controversial (62). There is also a high similarity of the common sequence EDFLVRSFYL (aa 847-856) of the IA-2 epitope peptides encompassing aa 841-898 with sequences in wheat and broad bean proteins. Peptide aa 841-856 contains a DR4 binding motif WCEDFLVRS (cf VLNDFLVRS in wheat and beans) and a predicted DQ8

binding motif IWCEDFLVRS (cf RVLNDFLVRS in wheat and beans). Antibodies to NADH reductase occur in some people with recently diagnosed diabetes. The class II MHC molecule of NOD mice, I-A^{g7}, is the structural counterpart of human DQ8(*0302), and NOD mice fed casein supplement (Harrison LC, unpublished), wheat flour and to a lesser extent soya bean meal (63), have an accelerated onset of diabetes.

In conclusion, the present inventors have identified T-cell epitope peptides in the intracytoplasmic domain of the type 1 diabetes autoantigen, tyrosine phosphatase IA-2, whose sequence analysis suggests that immunity to rotavirus (whose VP7 sequence mimics epitopes in both IA-2 and GAD) and possibly other viruses and dietary proteins, could predispose to type 1 diabetes by activating crossreactive T cells.

Our epidemiologic findings suggest that RV infection may trigger or exacerbate islet autoimmunity, on the HLA-DR4 background. As rotavirus vaccines currently undergoing trials are live viruses containing the VP7 leader sequence, our findings have implications for development of safe rotavirus vaccines to protect against islet autoimmunity and prevent the development of type 1 diabetes.

It will be appreciated by persons skilled in the art that numerous variations and/or modifications may be made to the invention as shown in the specific embodiments without departing from the spirit or scope of the invention as broadly described. The present embodiments are, therefore, to be considered in all respects as illustrative and not restrictive.

TABLE 1

Environmental agent	Peptide/Protein	aa	Experimental DR4 binding affinity(μM)	Sequence †	% Identity	% Similarity	#aas
				DR4 x x x x x DQ8 x x x x DQ8 x x x x			
	IA-2	805-820	2.5	V - V - - P - - F D G V F Q C			
Rotavirus A (serotype 3)	glycoprotein VP7	41-51		- - - - - - - - N A	56	100	9
Dengue virus	capsid protein C	104-112		- - - - I F T -	67	78	9
Cytomegalovirus (CMV, HHV5*)	major capsid	854-864		V G E - - - E - - - D	64	73	11
Canine distemper	haemagglutinin	89-99		- - - - - - - - K I I	75	88	8
Measles	haemagglutinin	88-96		- K D - - - - - K I I	63	75	8
Hepatitis C (strains J,T)	E2 genome polyprotein	374-384		- - - - - L F - G V D	45	64	11
Haemophilus influenzae	HI1338	96-110		V - D - - E V - K - - G Y K	50	71	14
				DR4 x x x x x DQ8 x x x x x			
	IA-2	685-700	9.0	A N M D - S - G - - - - Y M E			
Epstein-Barr (HHV4, strain B95-8)	BRRF2	179-188		- - - - - - - - A L R - - - - Q - -	71	86	7
Epstein-Barr (HHV4, strain B95-8)	BTRF1	93-102		- - - - - - - - - S L A - - - - - P	56	78	9
Rhinovirus 14 (common cold)	genome polyprotein	455-464		- - - - - - - - - - - - - - - -	50	100	10
				DR4 x x x x x DQ8 x x x x x			
	IA-2	787-802	10.0	- S H T - - - - - - - - W - - -			
Hantavirus	M polyprotein precursor	48-63		- P P V - - - - T A - - - - P - -	58	75	12
Japanese encephalitis	genome polyprotein	2956-2971		E A V D D P R - - E - - - D - E R	71	71	7
Kunjin, West Nile encephalitis,	genome polyprotein	~2956-2971		E A V D D P K - - E - - - D - E R	71	71	7
Murray Valley encephalitis	genome polyprotein	-2956-2972		E A V D D P K - - E - - - D - E R	71	71	7
				DR4 x x x x x DQ8 x x x x			
	IA-2	841-856	6.0	S E - I - C E - - - - - - - - L			
Wheat	NADH ubiquinone reductase	568-580		- - - R - L N - - - - - - - -	64	82	11
Vicia faba (broad bean)	NADH ubiquinone reductase	85-97		- - - R - L N - - - - - - - -	64	82	11
				DR4 x x x x x DQ8 x x x x x DQ8 x x x x x			
	IA-2	919-934	1.5	- - - - D - - - - - M A K G V K			
Herpes simplex (HHV1)	surface glycoprotein	2 to 9		- - - - T - - - - - - - - - -	63	88	8
Cow's milk	kappa casein	26-35		- - P - Q - - - - E - - - - -	60	80	10
				DR4 x x x x x DQ8 x x x x DQ8 x x x x			
	IA-2	859-874	1.0	- - - - - - - - - - V N A I L			
CMV (HHV5), H. saimiri	major capsid	1033-1043		P G - - - - - - - - R T D -	67	78	9
Papilloma 28,16	replication protein E1	392-402		- - - - - L - D T D A - - -	50	70	10
Vaccinia, Variola (HHV6)	E2L polyprotein	431-441		- - - - - K E - S D - - - -	45	73	11

*HHV = human herpes virus;

† potential anchor residues for binding to DR4 and DQ8 are denoted by x

TABLE 2: Epitopic peptides in IA-2 and homologous regions in IAR

Peptide #	T cell response: DR3,3;DQ2,2 control n=1	DR4,4;DQ8,8 control n=1	DR3,3;DQ2,2 relatives n=2	DR3,4;DQ2,8 relatives n=2	DR4,4;DQ8,8 relatives n=2	Position of sequence	Peptide sequence	Identity (%)	Homology (%)
15				1	2	IA-2 aa685-700 IAR aa721-736	ANMDISTGHMILAYME SNMDISTGHMILSYME	88	88
20					2	IA-2 aa713-728 IAR aa749-764	WQALCAYQAEPNTCAT WEALCAYQAEPNSSEV	69	75
26					2	IA-2 aa745-760 IAR aa781-796	PYDHARIKLKVESSPS TYDHSRVLLKAENSHS	56	69
34					2	IA-2 aa787-802 IAR aa823-838	LSHTTADFWQMVWESG LPATYADFWQMVWESG	81	88
35				1	2	IA-2 aa793-808 IAR aa829-844	DFWQMVWESGCTVIVM DFWQMVWESGCVVIVM	94	94
37	1		1	1	1	IA-2 aa799-814 IAR aa835-850	WESGCTVIVMLTPLVE WESGCVVIVMLTPLAE	88	94
38	1		2	2	2	IA-2 aa805-820 IAR aa841-856	VIVMLTPLVEDGVKQC VIVMLTPLAENGVRQC	81	94
45	1		1	1	2	IA-2 aa841-856 IAR aa877-892	SEHIWCEDFLVRSFYL SEHIWCEDFLVRSFYL	100	100
46					2	IA-2 aa845-860 IAR aa881-896	WCEDFLVRSFYLKNVQ WCEDFLVRSFYLKNLQ	94	100
47	1	1	1	1	2	IA-2 aa847-862 IAR aa883-898	EDFLVRSFYLKNVQTQ EDFLVRSFYLKNLQTN	88	100
54				1	1	IA-2 aa889-904 IAR aa925-940	DFRRKVNKCYRGRSCP DFRRKVNKCYRGRSCP	100	100
59			1	1	2	IA-2 aa919-934 IAR aa955-970	YTLIDMVLNRMAKGVK YVLIDMVLNKMAKGAK	81	100
66					2	IA-2 aa959-974 IAR aa995-1010	FEFALTAVAEEVNAIL FEFALTAVAEEVNAIL	100	100

TABLE 3

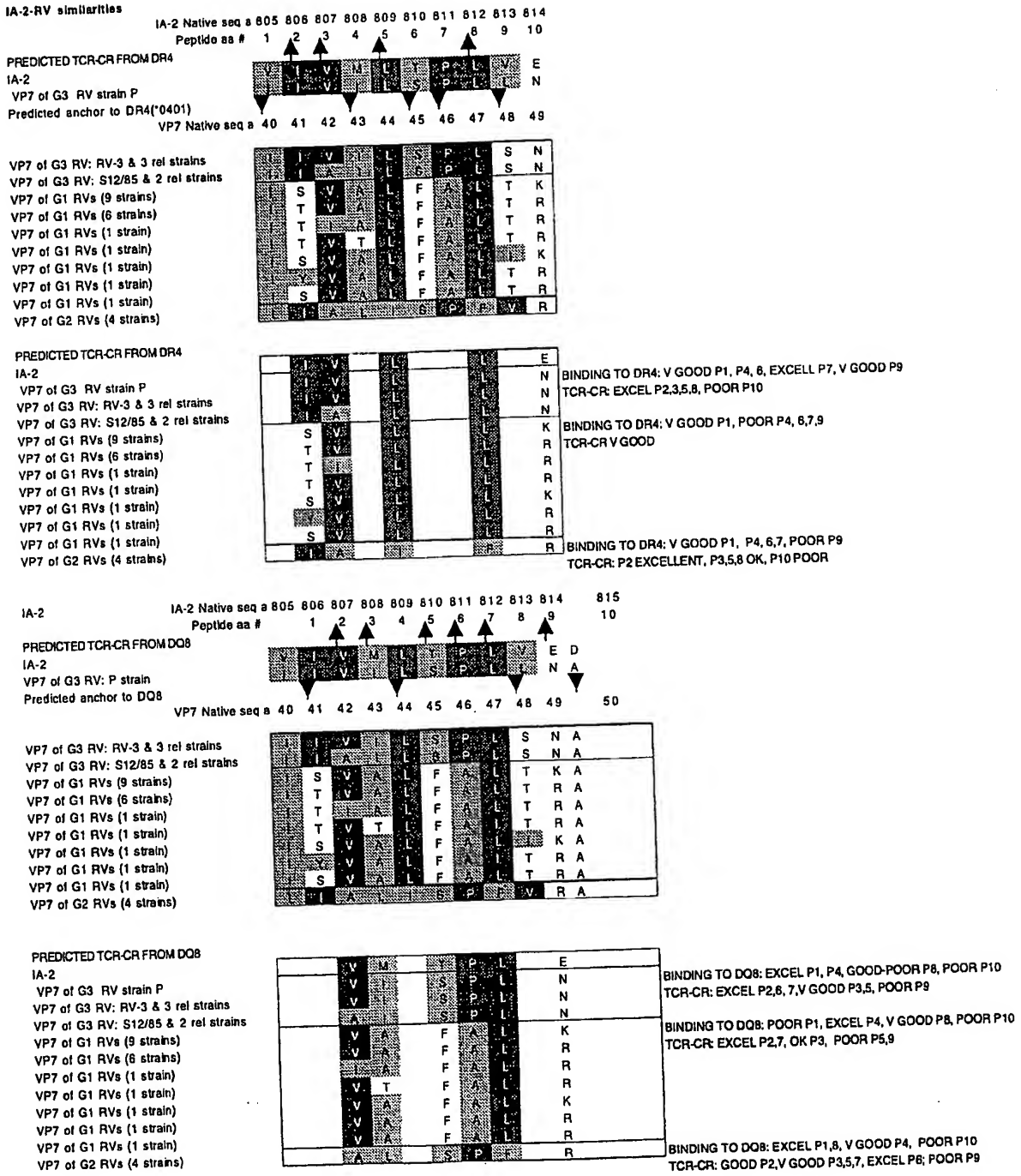

IA-2-RV similarities
IA-2 Native seq a 805 806 807 808 809 810 811 812 813 814
Peptide aa # 1 2 3 4 5 6 7 8 9 10
PREDICTED TCR-CR FROM DR4
IA-2
VP7 of G3 RV strain P
Predicted anchor to DR4(*0401)
VP7 Native seq a 40 41 42 43 44 45 46 47 48 49
VP7 of G3 RV: RV-3 & 3 rel strains
VP7 of G3 RV: S12/85 & 2 rel strains
VP7 of G1 RVs (9 strains)
VP7 of G1 RVs (6 strains)
VP7 of G1 RVs (1 strain)
VP7 of G1 RVs (1 strain)
VP7 of G1 RVs (1 strain)
VP7 of G1 RVs (1 strain)
VP7 of G1 RVs (1 strain)
VP7 of G2 RVs (4 strains)
PREDICTED TCR-CR FROM DR4
IA-2
VP7 of G3 RV strain P
VP7 of G3 RV: RV-3 & 3 rel strains
VP7 of G3 RV: S12/85 & 2 rel strains
VP7 of G1 RVs (9 strains)
VP7 of G1 RVs (6 strains)
VP7 of G1 RVs (1 strain)
VP7 of G1 RVs (1 strain)
VP7 of G1 RVs (1 strain)
VP7 of G1 RVs (1 strain)
VP7 of G1 RVs (1 strain)
VP7 of G2 RVs (4 strains)
BINDING TO DR4: V GOOD P1, P4, 6, EXCELL P7, V GOOD P9
TCR-CR: EXCEL P2,3,5,8, POOR P10
BINDING TO DR4: V GOOD P1, POOR P4, 6,7,9
TCR-CR V GOOD
BINDING TO DR4: V GOOD P1, P4, 6,7, POOR P9
TCR-CR: P2 EXCELLENT, P3,5,8 OK, P10 POOR
IA-2
IA-2 Native seq a 805 806 807 808 809 810 811 812 813 814 815
Peptide aa # 1 2 3 4 5 6 7 8 9 10
PREDICTED TCR-CR FROM DQ8
IA-2
VP7 of G3 RV: P strain
Predicted anchor to DQ8
VP7 Native seq a 40 41 42 43 44 45 46 47 48 49 50
VP7 of G3 RV: RV-3 & 3 rel strains
VP7 of G3 RV: S12/85 & 2 rel strains
VP7 of G1 RVs (9 strains)
VP7 of G1 RVs (6 strains)
VP7 of G1 RVs (1 strain)
VP7 of G1 RVs (1 strain)
VP7 of G1 RVs (1 strain)
VP7 of G1 RVs (1 strain)
VP7 of G1 RVs (1 strain)
VP7 of G2 RVs (4 strains)
PREDICTED TCR-CR FROM DQ8
IA-2
VP7 of G3 RV strain P
VP7 of G3 RV: RV-3 & 3 rel strains
VP7 of G3 RV: S12/85 & 2 rel strains
VP7 of G1 RVs (9 strains)
VP7 of G1 RVs (6 strains)
VP7 of G1 RVs (1 strain)
VP7 of G1 RVs (1 strain)
VP7 of G1 RVs (1 strain)
VP7 of G1 RVs (1 strain)
VP7 of G1 RVs (1 strain)
VP7 of G2 RVs (4 strains)
BINDING TO DQ8: EXCEL P1, P4, GOOD-POOR P8, POOR P10
TCR-CR: EXCEL P2,6, 7,V GOOD P3,5, POOR P9
BINDING TO DQ8: POOR P1, EXCEL P4, V GOOD P8, POOR P10
TCR-CR: EXCEL P2,7, OK P3, POOR P5,9
BINDING TO DQ8: EXCEL P1,8, V GOOD P4, POOR P10
TCR-CR: GOOD P2,V GOOD P3,5,7, EXCEL P6; POOR P9

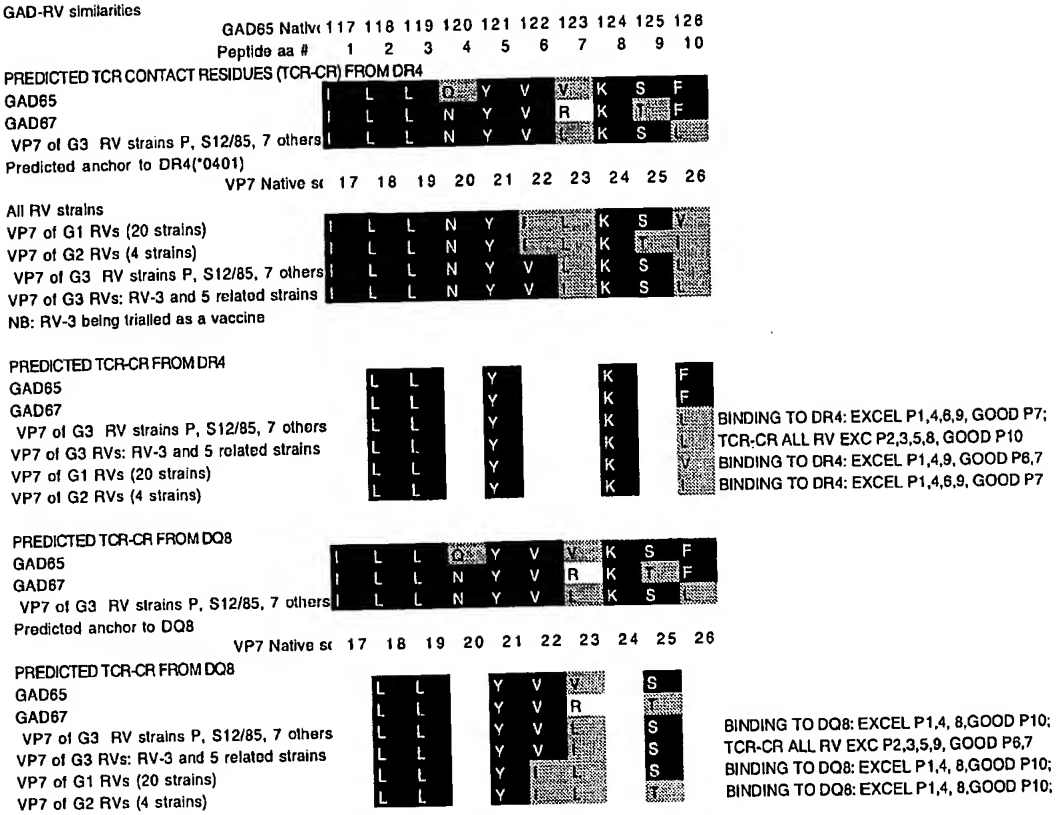
TABLE 4: sequence similarities between IA-2, rotavirus and GAD 65 and 67

Protein	Sequence †																																				
DR4			X			X		X	X		X															X			X		X	X		X			
DQ8			X			X				X		X														X			X				X		X		
IA-2 aa801-816	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	S	G	C	T	V	I	V	M	L	T	P	L	V	E	D	G
Rotavirus VP7 aa16-49	S	V	I	L	L	N	Y	V	L	K	S	L	T	R	I	M	D	F	I	I	Y	R	F	L	L	L	I	V	I	L	S	P	L	L			
GAD65 aa115-129	M	N	I	L	L	Q	Y	V	V	K	S	F	D	R	S																						
GAD67 aa121-135	V	D	I	L	L	N	Y	V	R	K	T	F	D	R	S																						

† potential anchor residues for binding to DR4 and DQ8 are denoted by x.

TABLE 5

GAD-RV similarities



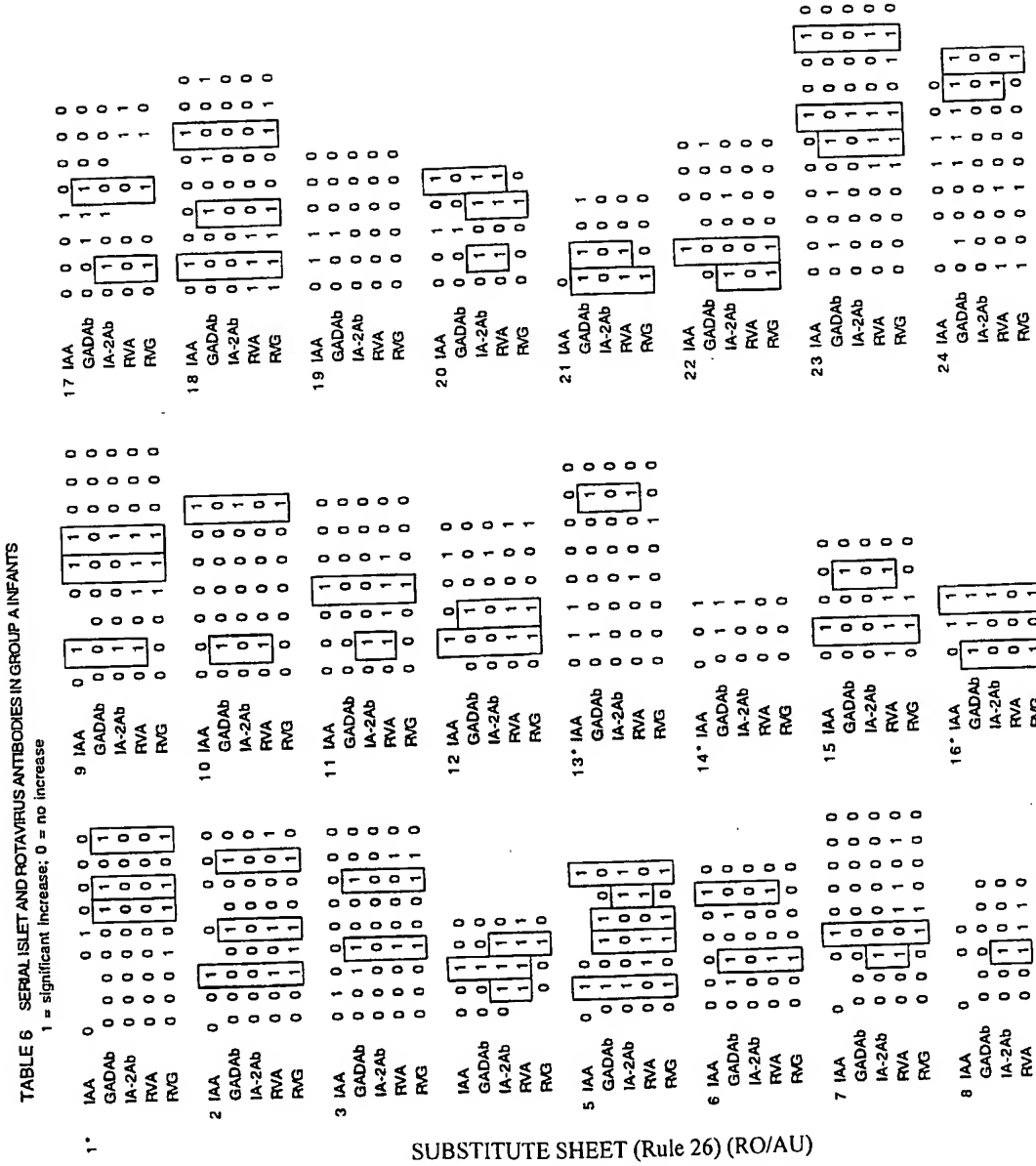


TABLE 6 SERIAL ISLET AND ROTAVIRUS ANTIBODIES IN GROUP A INFANTS
1 = significant increase; 0 = no increase
1*
2
3
4
5
6
7
8
9
10
11
12
13*
14*
15
16*
17
18
19
20
21
22
23
24
IAA
GADAb
IA-2Ab
RVA
RVG
* developed IDDM

TABLE 7. ISLET AND ROTAVIRUS ANTIBODIES IN ACUTE AND CONVALESCENT SERA COLLECTED AFTER ROTAVIRUS INFECTION*

INFANT		ACUTE	CONVALESCENT	INFANT		ACUTE	CONVALESCENT
1	IAA	nt	0.5	6	IAA	0.4	0.7
	GAD	1.6	0.2		GAD	0.1	0.1
	IA-2	0.7	0.8		IA-2	0.1	0.1
	RVA	6369	3724		RVA	0.1	24180
	RVG	72656	65383		RVG	2248	8411
2	IAA	0.4	0.7	7	IAA	1.1	1.1
	GAD	0.2	0.1		GAD	0.1	0.1
	IA-2	0.2	0.1		IA-2	0.1	0.1
	RVA	98900	10000		RVA	4407	nt
	RVG	4170000	>100000		RVG	5189	1567
3	IAA	0.4	1.1	8	IAA	0.3	1
	GAD	0.1	0.1		GAD	0.1	**14**
	IA-2	0.1	0.3		IA-2	0.1	**9.7**
	RVA	0.1	1026		RVA	7584	10001
	RVG	2462	39140		RVG	34560	>100000
4	IAA	nt	nt	9	IAA	0.3	0.6
	GAD	0.5	2.8		GAD	0.2	2.1
	IA-2	1.5	2.8		IA-2	0.1	**7.2**
	RVA	9673	55700		RVA	6337	4950
	RVG	33046	6390000		RVG	38500	86493
5	IAA	0.4	0.3	10	IAA	0.5	1.2
	GAD	**7.4**	4.9		GAD	0.1	0.1
	IA-2	**29**	**5.4**		IA-2	0.1	0.1
	RVA	2530	72200		RVA	3125	2888
	RVG	20980	>100000		RVG	50290	67083

*Convalescent sera were collected 4-8 weeks after gastroenteritis.

Significant levels of islet antibodies are bolded.

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CLAIMS:-

1. A tyrosine phosphatase IA-2 T-cell epitope, the T-cell epitope having a sequence included within or consisting of a sequence selected from the group consisting of ANMDISTGHMILAYME, WQALCAYQAEPNTCAT, PYDHARIKLKVESSPS, LSHTIADFWQMVWESG, DFWQMVWESGCTVIVM, WESGCTVIVMLTPLVE, VIVMLTPLVEDGVKQC, SEHIWCEDFLVRSFYL, WCEDFLVRSFYLKNVQ, EDFLVRSFYLKNVQTQ, DFRRKVNKCYRGRSCP, YILIDMVLNRMAKGVK, FEFALTAVAEEVNAIL and conservative substitutions therein.

2. A tyrosine phosphatase IA-2 T-cell epitope as claimed in claim 1 in which the T-cell epitope has a sequence included within or consisting of a sequence selected from the group consisting of ANMDISTGHMILAYME, WQALCAYQAEPNTCAT, PYDHARIKLKVESSPS, LSHTIADFWQMVWESG, DFWQMVWESGCTVIVM, WESGCTVIVMLTPLVE, VIVMLTPLVEDGVKQC, SEHIWCEDFLVRSFYL, WCEDFLVRSFYLKNVQ, EDFLVRSFYLKNVQTQ, DFRRKVNKCYRGRSCP, YILIDMVLNRMAKGVK, and FEFALTAVAEEVNAIL.

3. A tyrosine phosphatase IA-2 T-cell epitope as claimed in claim 1 or claim 2 in which the T-cell epitope has a sequence included within or consisting of the sequence VIVMLTPLVEDGVKQC.

4. A tyrosine phosphatase IA-2 T-cell epitope as claimed in any one of claims 1 to 3 in which the T-cell epitope has the sequence VIVMLTPLVED.

5. A T-cell epitope, the T-cell epitope having a sequence included within or consisting of a sequence selected from the group consisting of SNMDISTGHMILSYME, WEALCAYQAEPNSSFV, TYDHSRVLLKAENSHS, LPATVADFWQMVWESG, DFWQMVWESGCVVIVM, WESGCVVIVMLTPLAE, VIVMLTPLAENGVRQC, SEHIWCEDFLVRSFYL, WCEDFLVRSFYLKNLQ, EDFLVRSFYLKNLQTN, DFRRKVNKCYRGRSCP, YVLIDMVLNKMAKGAK, FEFALTAVAEEVNAIL and conservative substitutions therein.

6. A T-cell epitope as claimed in claim 5 in which the T-cell epitope has a sequence included within or consisting of a sequence selected from the group consisting of SNMDISTGHMILSYME, WEALCAYQAEPNSSFV, TYDHSRVLLKAENSHS, LPATVADFWQMVWESG, DFWQMVWESGCVVIVM, WESGCVVIVMLTPLAE, VIVMLTPLAENGVRQC,

SEHIWCEDFLVRSFYL, WCEDFLVRSFYLKNLQ, EDFLVRSFYLKNLQTN, DFRRKVNKCYRGRSCP, YVLIDMVLNKMAKGAK, and FEFALTAVAEEVNAIL.

7. A method of assessing the risk of an individual developing type 1 diabetes, the method comprising measuring responsiveness of T cells of the individual to at least one of the peptides as claimed in any one of claims 1 to 6 and comparing the T-cell responses from the individual without the peptides, and in comparison to responses from healthy controls.

8. A method for the preventionor delay of the onset of type 1 diabetes in an individual, the method comprising administering at least one peptide as claimed in any one claims 1 to 6 to the individual to induce tolerance to the epitope.

9. A method as claimed in claim 8 in which the at least one peptide is administered to a mucosal surface of the subject.

10. A method as claimed in claim 9 in which the peptide is administered orally, by aerosol or intranasally.

11. A method of preventing or delaying the re-establishment of type 1 diabetes in an individual who has received a pancreatic or islet-cell transplant, the method comprising administering at least one peptide as claimed in any one claims 1 to 6 to the individual to induce tolerance to the epitope.

12. A method as claimed in claim 11 in which the at least one peptide is administered to a mucosal surface of the subject.

13. A method as claimed in claim 12 in which the peptide is administered orally, by aerosol or intranasally.

14. A vaccine composition for use in raising an immune response in a subject directed against rotavirus, the composition comprising a plurality of antigens including at least one rotavirus VP7 antigen wherein the sequence of the rotavirus VP7 antigen is modified such that at least one of the sequences which mimic one or more of the epitopes selected from the group consisting of ILLQYVVKSFDRS, ILLNYVRKTFDRS and VIVMLTPLVED are deleted or modified such as to remove or ameliorate mimicry.

15. A vaccine composition as claimed in claim 14 in which the composition comprises at least one attenuated strain of rotavirus, the rotavirus nucleic acid encoding VP7 being modified such that the expressed VP7 antigen is modified such that at least one of the sequences which mimic

one or more of the epitopes selected from the group consisting of ILLQYVVKSF, ILLNYVRKTF and VIVMLTPLVED are deleted or modified such as to remove or ameliorate mimicry.

16. A vaccine composition as claimed in claim 14 or claim 15 in which the the sequence which mimics ILLQYVVKSF or ILLNYVRKTF is deleted.

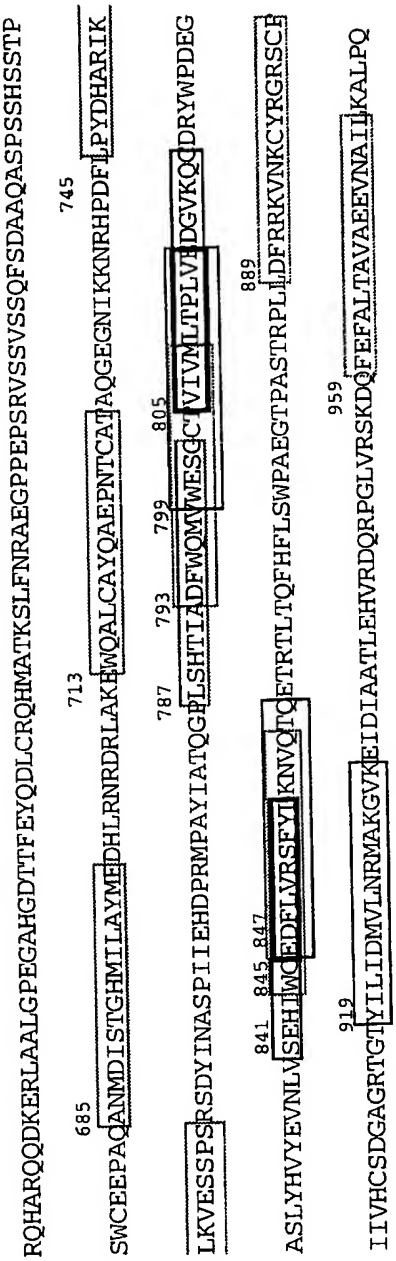
17. A ligand for antigen-specific T lymphocytes, the ligand comprising a multimeric peptide-MHC complex in which the peptide is selected from the group consisting of ANMDISTGHMILAYME, WQALCAYQAEPNTCAT, PYDHARIKLKVESSPS, LSHTIADFWQMVWESG, DFWQMVWESGCTVIVM, WESGCTVIVMLTPLVE, VIVMLTPLVEDGVKQC, SEHIWCEDFLVRSFYL, WCEDFLVRSFYLKNVQ, EDFLVRSFYLKNVQTQ, DFRRKVNKCYRGRSCP, YILIDMVLNRMAKGVK, FEFALTAVAEEVNAIL, SNMDISTGHMILSYME, WEALCAYQAEPNSSFV, TYDHSRVLLKAENSHS, LPATVADFWQMVWESG, DFWQMVWESGCVVIVM, WESGCVVIVMLTPLAE, VIVMLTPLAENGVRQC, SEHIWCEDFLVRSFYL, WCEDFLVRSFYLKNLQ, EDFLVRSFYLKNLQTN, DFRRKVNKCYRGRSCP, YVLIDMVLNKMAKGAK, FEFALTAVAEEVNAIL and conservative substitutions therein.

18. A ligand as claimed in claim 17 in which the peptide is VIVMLTPLVEDGVKQC or VIVMLTPLVED.

19. A ligand as claimed in claim 17 or 18 in which the multimer is a dimer or tetramer.

20. A ligand as claimed in any one of claims 17 to 20 in which the ligand is provided with a detectable label, such as biotin, a fluorophor, or radioisotope.

Figure 1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787
793
799
805
LKVESSPSRSDYINASPIIEHDPRMPAYIATQGPLSHTIADFWQMVWESGCTVIVMLTPLVEDGVKQCDRYWPDEG
841
845
847
889
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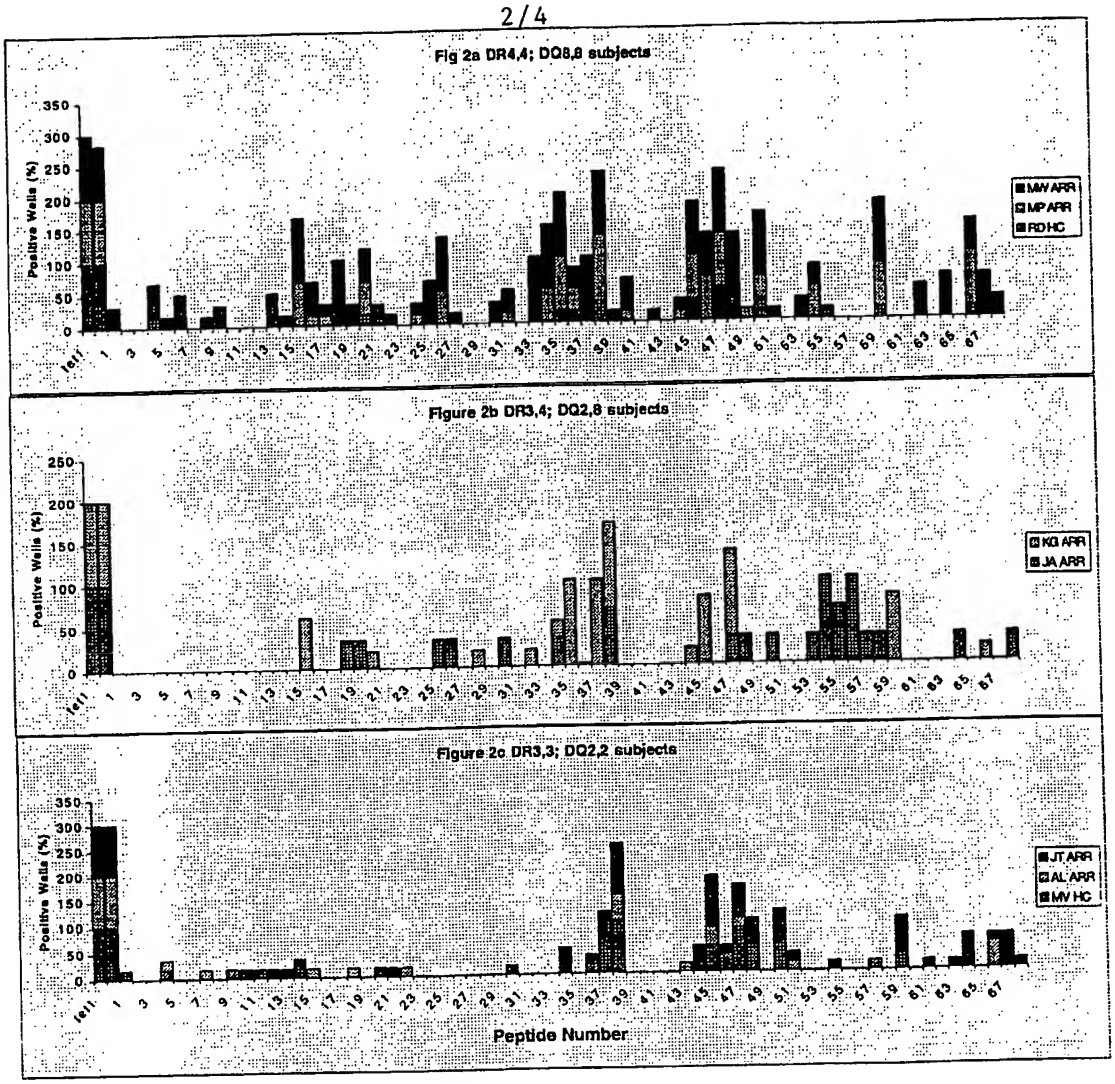
Fig 2a DR4,4; DQ8,8 subjects
Positive Wells (%)
MV ARR
MP ARR
RD HC
Figure 2b DR3,4; DQ2,8 subjects
Positive Wells (%)
KG ARR
JA ARR
Figure 2c DR3,3; DQ2,2 subjects
Positive Wells (%)
JT ARR
AL ARR
MV HC
Peptide Number

FIGURE 2

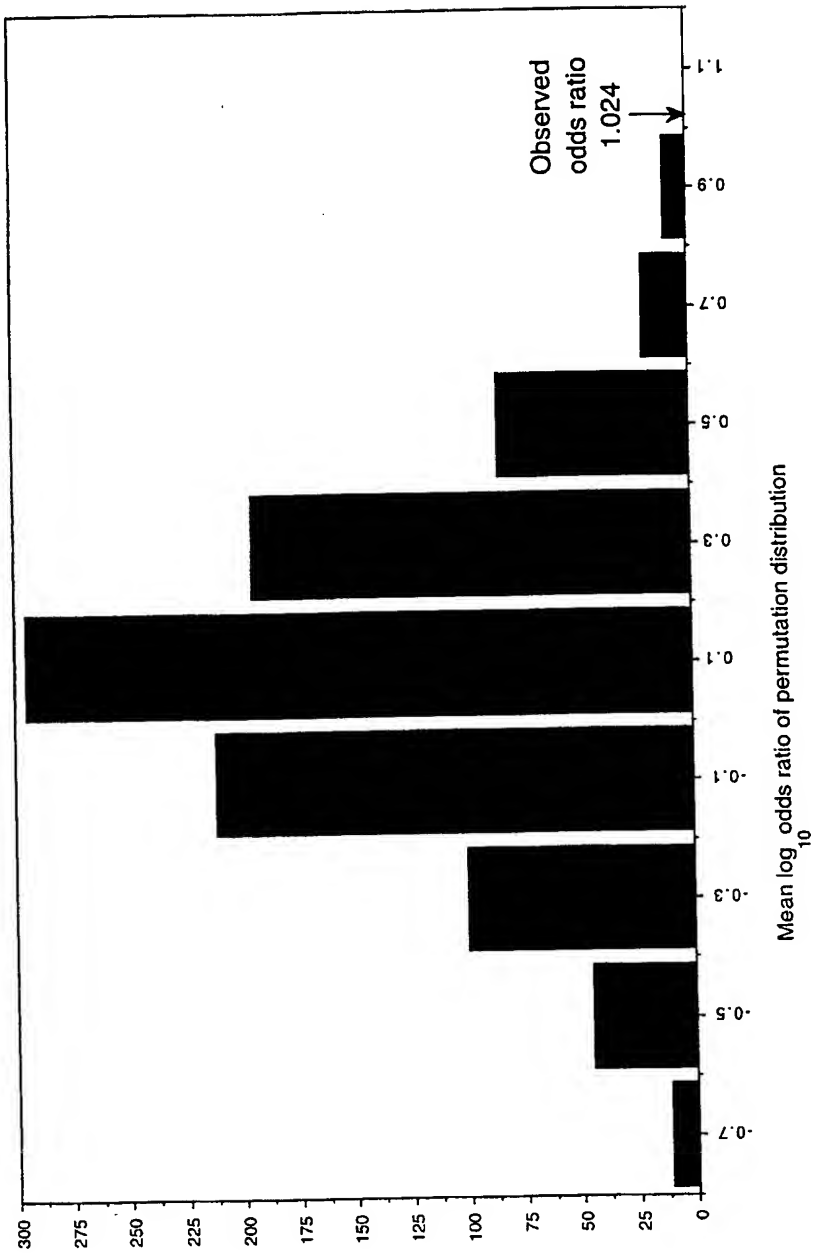
Observed odds ratio 1.024
1.1
0.9
0.7
0.5
0.3
0.1
-0.1
-0.3
-0.5
-0.7
Mean log10 odds ratio of permutation distribution
300
275
250
225
200
175
150
125
100
75
50
25
0

FIGURE 3

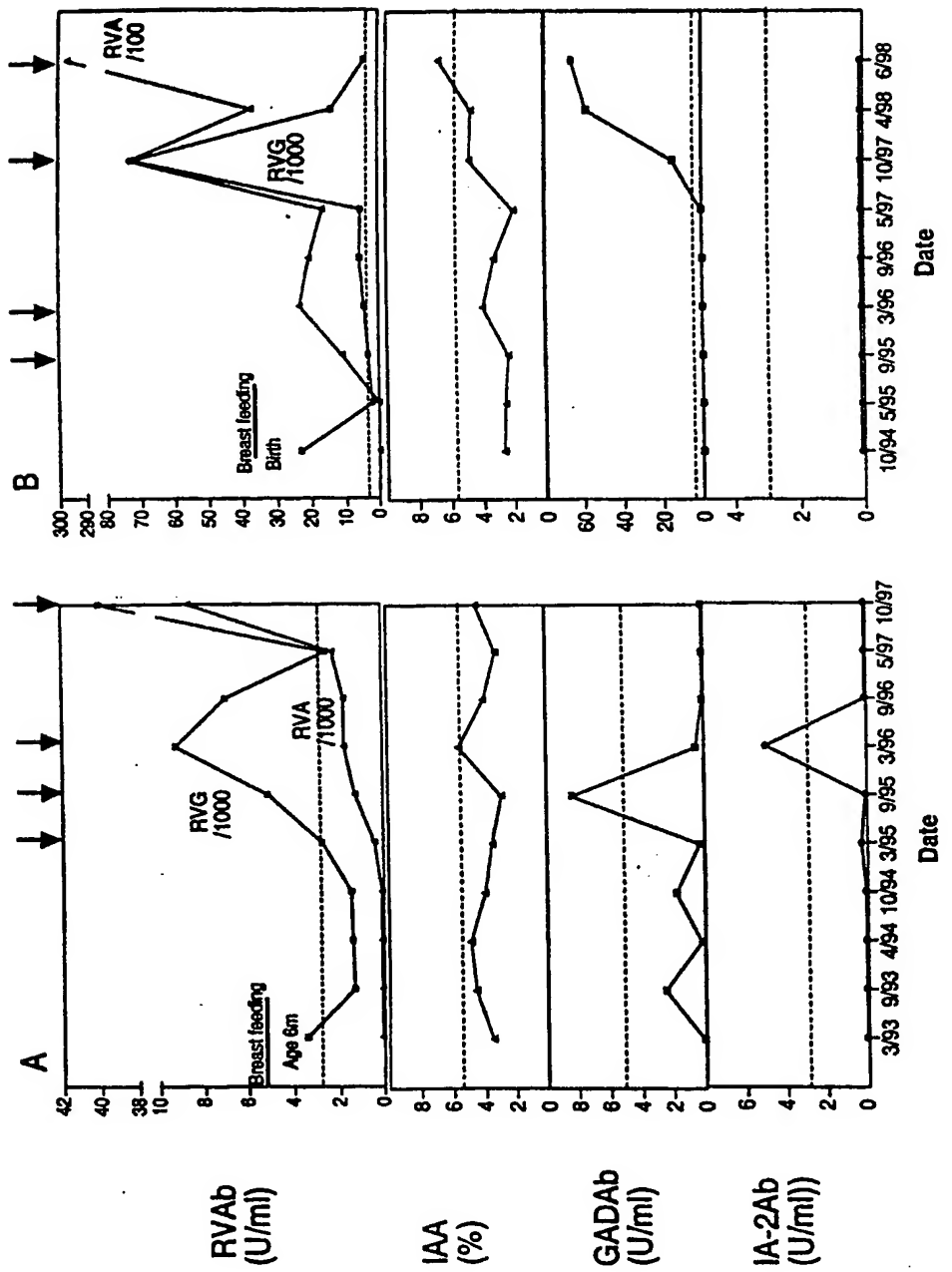
A
B
RVG /1000
RVA /1000
RVA /100
Breast feeding
Age 6m
Birth
RVAb (U/ml)
IAA (%)
GADAb (U/ml)
IA-2Ab (U/ml))
Date
3/93 9/93 4/94 10/94 3/95 9/95 3/96 9/96 5/97 10/97
10/94 5/95 9/95 3/96 9/96 5/97 10/97 4/98 6/98

FIG. 4

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU 99/00314

A. CLASSIFICATION OF SUBJECT MATTER

Int Cl[6]: C12N 9/12 C07K 7/00 A61K 39/15, 37/02

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
WPAT, Chemical Abstracts - See below[6]

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
Medline - See below

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
WPAT, Chemical Abstracts, Medline: protein tyrosine phosphatase, tyrosine protein phosphatase, tyrosine phosphatase, epitope, diabetes, 1A-2, VP7

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P, X	Nature Biotechnology (1998, October) volume 16, pages 966-969 by Honeyman MC et al. "Neural network-based prediction of candidate T-cell epitopes". See entire document.	1-20
X	Molecular Medicine (1997) volume 29, pages 401-404 by Honeyman MC et al. "Strategies for identifying and predicting islet autoantigen T-cell epitopes in insulin-dependent diabetes mellitus". See entire document, in particular Methods section.	1-20
X	Molecular Medicine (1998, April) volume 4, pages 231-9 by Honeyman MC et al. "T-cell epitopes in type 1 diabetes autoantigen tyrosine phosphatase IA-2: potential for mimicry with rotavirus and other environmental agents". See entire document.	1-20

[X] Further documents are listed in the continuation of Box C

[ ] See patent family annex

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search
10 June 1999

Date of mailing of the international search report
22 JUN 1999

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU 99/00314

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Diabetes (1997) volume 46, pages 40-43 by Zhang B et al. "Autoantigens to IA-2 in IDDM: location of major antigenic determinants". See entire document.	1-20
A	Journal of Immunology (1996) volume 157, pages 2707-2711 by Lampasona V et al. "Autoantibodies in Insulin-dependent diabetes recognize distinct cytoplasmic domains of the protein tyrosine phosphatase-like IA-2 autoantigen". See entire document.	1-20